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Characterization and comparative analysis of a second thermonuclease from *Staphylococcus aureus*

Yu Hu[a], Jianghong Meng[b], Chunlei Shi[a], Kirstin Hervin[a], Pina M. Fratamico[c], Xianming Shi[a,*]

[a] MOST-USDA Joint Research Center for Food Safety & Bor Luh Food Safety Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, PR China
[b] Joint Institute for Food Safety and Applied Nutrition, Department of Nutrition and Food Science, University of Maryland, College Park, MD 20742, USA
[c] U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA 19038, USA

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ABSTRACT

Staphylococcal nuclease (here termed as Nuc1) is considered an important virulence factor and a unique marker widely used in the detection of *Staphylococcus aureus*. A second functional thermostable nuclease (here termed as Nuc2) in *S. aureus* was characterized after recombinant expression in *Escherichia coli*. Sequence alignment and phylogenetic analysis revealed that Nuc2 was a more conserved protein in the staphylococci group compared with Nuc1. Recombinant Nuc2 showed nuclease activity in the zymogram test and was able to degrade various types of nucleic acids. The optimal reaction temperature and pH for Nuc2 were 50 °C and pH 10, respectively. The enzymatic activity of Nuc2 was stimulated in the presence of Ca^{2+} (0.05 mM), Mg^{2+} (0.5 mM), dithiothreitol, β-mecaptoethanol, TritonX-100, Tween-20, and urea; however, activity decreased sharply when exposed to heavy metals such as Zn^{2+} and Mn^{2+}, and in the presence of EDTA or SDS. Nuc2 showed weaker activity, lower thermostability and different sensitivity to these chemical agents compared with Nuc1, which was consistent with differences in the sequence pattern and structure predicted. Furthermore, a *nuc1* and *nuc2* double deletion mutant of *S. aureus* and respective complementation experiments suggest a major role for *nuc1* in terms of thermonuclease activity in *S. aureus*.

1. Introduction

Staphylococcal nuclease is considered as an important virulence factor and a unique marker widely used in the detection of *Staphylococcus aureus* from food samples and clinical specimens (Sandel and McKillip 2004; Alarcon et al. 2006). It is an exoenzyme that could hydrolyze DNA and RNA in host cells, causing tissue destruction and spreading of *S. aureus* (Sandel and McKillip 2004; Foster 2005). It was also the first indication of a new class of sugar-nonspecific nucleases because of its high level of extracellular production and remarkable heat stability in *S. aureus* (Tucker et al. 1978). Sugar non-specific nucleases are characterized by their ability to hydrolyze both DNA and RNA without exhibiting pronounced base preferences (Rangarajan and Shankar 2001). They play important roles in DNA and RNA degradation, which is essential in microbial defense mechanisms and programmed cell death (Hsia et al. 2005; Parrish and Xue 2006).

Staphylococcal nuclease is one of a few sugar non-specific nucleases that have been extensively characterized (Tucker et al. 1979; Cole et al. 1988). This enzyme is a 5′-phosphodiesterase that cleaves either single- or double-stranded DNA or RNA generating 3′-nucleotides, dinucleotides, and particularly, thermostable phosphates (Anfinsen 1968). The mature extracellular form of the enzyme is a single polypeptide chain consisting of 149 amino acids. This monomeric protein lacks disulfide bridges and can refold spontaneously after thermal unfolding, thus it was also used as a model to study protein-folding mechanisms (Gamble et al. 1994; Shortle and Meeker 1989; Su et al. 2005). In addition, it is ideal for the biotechnology industry for removing heterologous nucleic acids due to its promiscuous ability to hydrolyze chromosomal DNA, plasmid DNA, and RNA (Cooke et al. 2003).

The second thermonuclease of *S. aureus* had been predicted based on the whole genome sequence data of *S. aureus* (Kuroda et al. 2001). Previous studies have verified the existence of these two functional thermostable nucleases encoded by *nuc1* and *nuc2* in *S. aureus* (Tang et al. 2008), and comparative expression analysis of these two genes in *S. aureus* has also been investigated recently (Hu et al. 2012). However, the characteristic and evolutionary difference of the second thermonuclease from *S. aureus* remains unknown. In addition, the nomenclature of the thermonuclease gene in *S. aureus* is still obscure because researchers often use the gene name *nuc* for either *nuc1* or *nuc2* indiscriminately, which makes the detection, diagnosis, or other applications of

* Corresponding author. Fax: +86 21 3420 6616.
E-mail address: xmshi@sjtu.edu.cn (X. Shi).

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thermonuclease in *S. aureus* confusing. Therefore, research on the relatedness and differences in the second thermonuclease (Nuc2) with staphylococcal nuclease is a pressing need. In the present study, the two thermonucleases of *S. aureus* were compared using sequence alignment and phylogenetic analysis. Their nuclease activities were further characterized after expressed in *Escherichia coli*. Furthermore, a *nuc1* and *nuc2* double deletion mutant of *S. aureus* was constructed by homologous recombination, and complementation experiments were then carried out. Thermonuclease assays revealed functional activity differences of *nuc1* and *nuc2* in *S. aureus*. The purpose of this study was to determine important characteristics of the second thermonuclease (Nuc2) from *S. aureus* and shed light on evolutionary and enzymatic differences of Nuc1 and Nuc2 by comparative analysis.

2. Materials and methods

2.1. Amino acid sequences and phylogenetic analysis

The sequences of *nuc1* (SA0746) and *nuc2* (SA1160) in *S. aureus* were obtained from the *S. aureus* N315 genome in GenBank. The signal peptides of Nuc2 and the hydrophobic regions were detected by SignalP 3.0 (Bendtsen et al. 2004). Alignment of the deduced amino acid sequences was generated by the Cluster W program (Larkin et al. 2007). The sequences of NUC proteins from different species were obtained from blastp searching and were assembled using BioEdit 7.0.5 with the Clustal W package (Hall 1999). The phylogenetic tree of Nuc proteins was generated by the MEGA 4.0 package using the Neighbor-joining (NJ) algorithm (http://www.megasoftware.net) with Poisson correction and Bootstrap values at 1000.

2.2. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used for this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium with the addition of ampicillin (100 μg/ml) as needed at 37 °C. *S. aureus* strains were grown in Tryptic Soy Broth (TSB) medium and brain heart infusion (BHI) medium with aeration at 37 °C or 30 °C for strains harboring thermo-sensitive plasmids. Antibiotics were used at the following concentrations where appropriate: chloramphenicol (Cm), 20 μg/ml; kanamycin (Kan), 50 μg/ml.

2.3. Recombinant expression of Nuc1 and Nuc2

Recombinant expression of Nuc1 and Nuc2 was performed according to previously reported methods (Tang et al. 2008). Briefly, the coding sequences of *nuc1* (696 bp) and *nuc2* (656 bp) were cloned into pET-28a. The recombinant plasmids and the empty plasmid were separately transformed into *E. coli* BL21 (DE3) by the $CaCl_2$ method and the transformant was grown in Luria-Bertani broth (Difco, BD, Franklin Lakes, NJ, USA) with kanamycin (50 μg/ml) at 37 °C. Expression of the target proteins was induced for 6 h by adding isopropyl β-D-1-thiogalactoside (IPTG) when the cells had reached an OD600 of 0.6–0.8. *E. coli* BL21 (DE3) with plasmid pET-28a was used as a negative control in all the experiments described below. Expression was analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the same amount of whole cell lysates with similar protein levels. The soluble protein was isolated for further characterization.

2.4. In situ nuclease activity/zymogram gels

Whole cell proteins from the recombinant *E. coli* after induction were processed for *in situ* staining of nuclease activity according to Brnakova et al. (2005) with modifications. Briefly, samples from expression analysis were separated by 12% SDS-PAGE containing herring sperm DNA (Sigma–Aldrich, USA) at a final concentration 10 μg/ml. After separation, gels were washed at room temperature with three changes (100 ml for 15 min each) of 40 mM Tris–HCl buffer (pH 7.6) on an orbital shaker. Subsequently, the gel was incubated in 40 mM Tris–HCl pH 7.6, 2 mM $MgCl_2$, 2 mM $CaCl_2$ buffer overnight at 30 °C with gentle agitation to allow enzyme digestion. The gel was then rinsed with deionized water and stained with 1 μg/ml of ethidium bromide. Nuclease activity was determined under UV light by visualizing clear bands following substrate hydrolysis.

2.5. Substrates for the recombinant nucleases

To determine the specificity of substrate for the recombinant nuclease, genomic DNA extracted from *S. aureus*, *Listeria monocytogenes*, and *Salmonella*, plasmid DNA pNucc from *S. aureus* and pBR122 from *E. coli*, and RNA from *S. aureus* were treated with the recombinant Nuc1 and Nuc2 in 20 μl of reaction buffer at 37 °C for 6 h, respectively. This was followed by analysis of 10 μl of the reaction solution by electrophoresis on a 1.0% agarose gel.

2.6. Thermonuclease activity assay

The soluble protein from recombinant expression was heated at 100 °C for 20 min, and supernatants were tested for nuclease activity. A spectrophotometric assay was carried out according to Man and Chesbro (1980) with modifications. Briefly, diluted boiled sample aliquots were placed in a 50 °C water bath, followed by addition of 20 μl of 2 mg/ml heat denatured salmon sperm DNA and 20 μl of 50 mM Tris buffer (pH 9.0) with 1 mM $CaCl_2$. After 30 min incubation, test reactions were stopped by adding an equal volume of 10% ice-cold perchloric acid. The solutions were held on ice for 10 min, and 0.9 ml of cold distilled water was added to each tube. The samples were centrifuged at 8000 × *g* for 5 min at 4 °C. The optical density of the supernatants was measured at 260 nm using a DU-800 spectrophotometer. One unit of thermonuclease activity was equivalent to an increase at A260 nm of 0.5 compared with the negative control under the aforementioned reaction conditions. The time course of nuclease activity of recombinant Nuc1 and Nuc2 was tested every 3–10 min.

2.7. Effects of temperature and pH on nuclease activity of Nuc1 and Nuc2

An enzymatic activity assay was performed to measure the effects of temperature on the nucleases at 20, 30, 37, 45, 50, 55, 60, 70, and 80 °C using herring sperm DNA as substrate (Sigma–Aldrich, St. Louis, MO, USA). The effect of pH on nuclease activity was evaluated at pH values ranging from 3.0 to 11.0 using the optimal temperature determined above. These assays and those described below were all conducted in triplicate.

2.8. Effects of metal ions, metal chelators and other compounds on nuclease activity of Nuc1 and Nuc2

The effects of metal ions on the nuclease activity of Nuc1 and Nuc2 were determined by treatment with eight different metal ions, Ca^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and K^{+}, at 50 °C for 30 min. The concentrations of metal ions in the final nuclease reactions were 0.05, 0.5, or 5 mM. To determine the effects of metal chelators, thiol reagents, and detergents, the nuclease was incubated with different concentrations of these agents followed by the nuclease activity assay. The concentrations of ethylene diamine tetraacetic acid (EDTA), citrate, dithiothreitol (DTT), β-mecaptoethanol (2-ME), and urea were 1 mM or 10 mM in the

Table 1
Bacterial strains, plasmids and primers used in this study.

Strains	Key features[a]	Source/reference
Strains		
E. coli		
TG1	supEhsdΔ5 thiΔ(lac-proAB) F′[traD36 proAB+lacIqlacZΔM15], for construction of recombinant plasmid	Laboratory stock
BL21(DE3)	Host strain for expression of recombinant plasmid	Laboratory stock
S. aureus		
RN4220	A derivative of NCTC 8325-4 that accepts foreign DNA	Dr. Karen Battista
RNΔ*nuc1*	RN4220, *nuc1*::Em; Em^r	Tang et al. (2008)
RNΔ*nuc2*	RN4220, *nuc2*::Kan; Kan^r	Unpublished data
RNΔ*nuc1nuc2*	RN4220, *nuc1*::erm, *nuc2*::Kan; $Kan^r Em^r$	This work
Plasmids		
pET-28a	A prokaryotic expression vector, kanamycin resistant	Novagen
pET-28a-nuc1	pET-28a carrying a *nuc1* expression cassette	This work
pET-28a-nuc2	pET-28a carrying a *nuc2* expression cassette	This work
pBT2	$Cm^r Amp^r$; thermosensitive *E. coli–S. aureus* shuttle vector	Dr Reinhold Bruckner
pPIC9K	Source of the Kan^r cassette	Laboratory stock
pBT2Δnuc2	pBT2 carrying a kan cassette flanked by the upstream and downstream regions of *nuc2* gene in the genome of *S. aureus*	This work
pBT2nuc2-C	pBT2 carrying a *nuc2* expression cassette	This work
pBT2nuc1-C	pBT2 carrying a *nuc1* expression cassette	Tang et al. (2008)
Primer		
Nuc2F-EcoRI	5′-CGAGAATTCAGTATTTCACAGCGTGTAC-3′	This work
Nuc2F-BamHI	5′-ATAGGATCCTATTGCCACCTACTTCTC-3′	This work
Nuc2B-BamHI	5′-CTCTGGATCCATAGTGCGTATCGAGCGTA-3′	This work
Nuc2B-PstI	5′-CAGCTGCAGTCACGAATCTTTCACGTCA-3′	This work
KANH-1	5′-GATGGATCCGGGGGGGAAAGCCACGTTG-3′	This work
KANH-2	5′-GCTGGATCCTGCCTCGTGAAGAAGGTGTTGC-3′	This work
Nuc2-C1	5′-GTACGCTGCAGACGAAACAATCAAGTAAACG-3′	This work
Nuc2-C2	5′-CTAGGAATTCTCGATACGCACTATGGTTAT-3′	This work

[a] *Abbreviations*: Em^r, erythromycin resistance; Kan^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance.

incubation solution and 0.5 mM or 5 mM in the final nuclease reaction, respectively. The concentrations of SDS, TritonX-100, and Tween-20 were 0.1% or 1% (w/v) in the incubation solution and 0.05% or 0.5% in the final nuclease reaction, respectively.

2.9. Comparative structural analysis

The protein domain composition and active site of both Nuc1 and Nuc2 were analyzed by InterPro database (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The homology model of Nuc2 was constructed by the web server 1.0 of ESyPred3D (Lambert et al. 2002) using the crystal structure of Nuc1 (PDB entry:1SNP) as the template. The structures of these two proteins were superimposed, and the figures were drawn by the VMD package (Humphrey et al. 1996).

2.10. Construction of nuc1 and nuc2 double deletion mutant in S. aureus

S. aureus RNΔ*nuc1 nuc2* double deletion mutant was constructed by electroporation of *nuc2* deletion mutation plasmid (pBT2Δ*nuc2*) into the *nuc1* mutant of *S. aureus* RN4220. The *nuc1* deletion strain *S. aureus nuc1::erm* was constructed previously (Tang et al. 2008). Deletion of the *S. aureus nuc2* gene was performed by homologous recombination using the temperature-sensitive shuttle plasmid pBT2Δ*nuc2* in which the chromosomal *nuc2* gene (∼540 bp) was deleted and replaced by a kanamycin resistance marker kan (∼1.2 kb). Briefly, the upstream 1141-bp fragment of the *nuc2* gene was amplified from the genomic DNA of *S. aureus* strain RN4220 with the primers Nuc2F-*Eco*RI and Nuc2F-*Bam*HI. The PCR fragment was digested with *Eco*RI and *Bam*HI and then cloned into the shuttle vector pBT2, resulting in a recombinant plasmid pBT2-nuc2F. The downstream 1072-bp fragment of the *nuc2* gene was amplified with the primers Nuc2B-*Bam*HI and Nuc2B-*Pst*I and cloned between the *Bam*HI and *Pst*I restriction sites of pBT2-nuc2F. A 1.2 kb kan cassette (*kan*) that was amplified from the plasmid pPIC9K with the primers KANH-1 and KANH-2, was digested with *Bam*HI, and then ligated into the *Bam*HI site of the recombinant plasmid to generate the final knockout plasmid pBT2Δ*nuc2* for homologous recombination. The shuttle plasmid pBT2Δ*nuc2* was isolated from *E. coli* TG1 and introduced into *S. aureus* RNΔ*nuc1* mutant by electroporation and selected on BHI agar containing chloramphenicol (Cm) (20 μg/ml). A positive transformant was grown in BHI medium (Cm) (20 μg/ml) at 30 °C overnight. Subsequently, the culture was diluted 1000-fold at 42 °C in BHI medium with kanamycin and cultured for six passages. Then, the culture was diluted 1000-fold in BHI medium without selection at 42 °C to facilitate selection of the second recombination event. Cultures were plated at 42 °C on BHI agar containing kanamycin. After 24 h, the kan-resistant and Cm-sensitive colonies were identified as ones in which replacement recombination occurred. Correct *nuc2* gene inactivation was confirmed by PCR and Southern blotting.

2.11. Complementation of RNΔnuc1 nuc2 and the impact on thermonuclease activity

The upstream 955-bp fragment of the *nuc2* gene was amplified from the genomic DNA of *S. aureus* strain RN4220 using primers Nuc2-C1 and Nuc2-C2. The PCR products were then subcloned into the shuttle vector pBT2 in *E. coli* TG1, and subsequently transformed to the *S. aureus* RNΔ*nuc1 nuc2* by electroporation. Meanwhile, the *nuc1* complementing plasmid was also transformed into the double deletion mutant to complement the *nuc1* deletion. The mutants and the complementation strains were grown in TSB medium overnight and then heated at 100 °C for 20 min. The supernatants were tested for nuclease activity using toluidine blue DNA agar according to the method described previously (Hu et al. 2012).

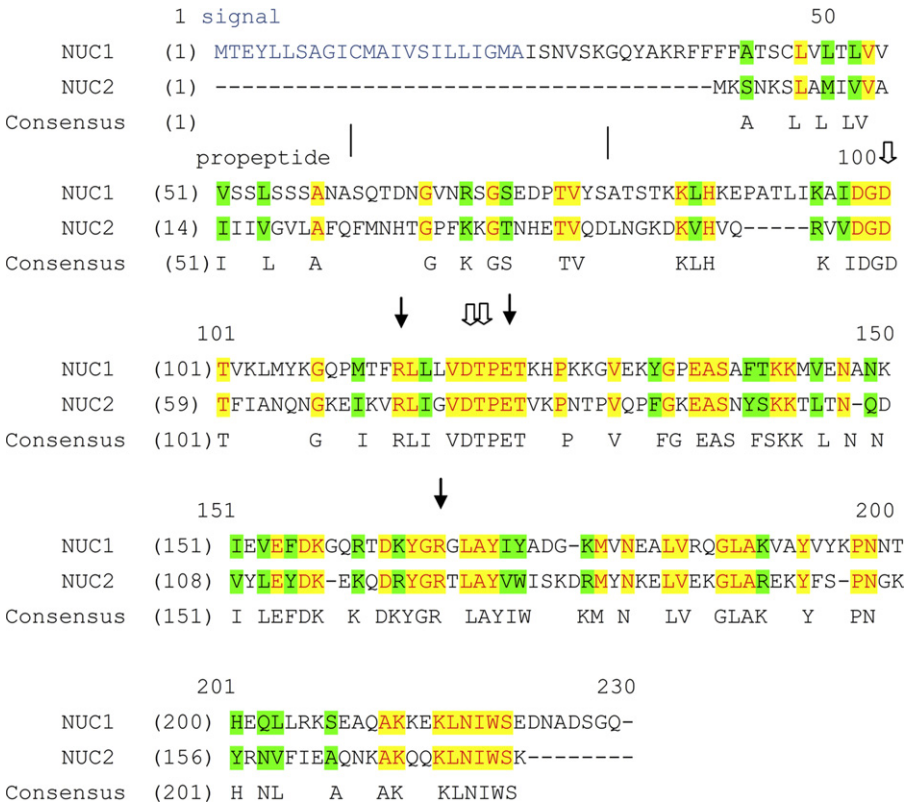

Fig. 1. Alignment of deduced amino acid sequences of two thermonucleases in *S. aureus*. The conserved amino acid residues are shown in red with yellow background, less conserved residues appear with green background. Solid arrows indicate conserved active site Arg114, Glu122, Arg166, while the hollow arrows indicate metal binding site Asp 100, Asp119, Thr120. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results

3.1. Alignment and evolutionary relationships of Nuc1 and Nuc2

The amino acid sequences derived from *nuc1* (687 bp) and *nuc2* (534 bp) showed that Nuc1 and Nuc2 were both a single polypeptide chain consisting of 228 and 177 amino acids, respectively (Fig. 1). There were hydrophobic regions at the N-terminus of both enzymes, and these regions are considered as signal peptides. Nuc1 has a long signal peptide (60 amino acids), containing two hydrophobic stretches, which was cleaved off during secretion (Suciu and Inouye 1996), whereas only 25 amino acids were predicted as a signal peptide with only one hydrophobic stretch in Nuc2. Sequence alignment revealed that Nuc2 shared 34.6% identity with Nuc1; however, the residues constituting the active site were conserved (Cotton et al. 1979), including the "DTPE" motif, which contains a highly conserved glutamic acid at the active site and two metal binding sites (Fig. 1).

Nucleotide blast analysis revealed no significant similarity between *nuc1* and *nuc2* except 28 identical nucleotides at their 3′ ends (Tang et al. 2008). In contrast to *nuc1*, which is quite specific to *S. aureus*, we found that the *nuc2* gene had 79% identity with the *nuc* gene in *Staphylococcus epidermidis*, suggesting a close relationship of *nuc2* with similar genes in other species in the genus *Staphylococcus*. To gain better insight on the evolution of the thermonuclease and to determine the relatedness of the two thermonucleases in *S. aureus*, aphylogenetic tree of closely related thermonucleases from 24 different bacterial species was constructed. Interestingly, Nuc2 and Nuc1 were found located in two different clusters (Fig. 2). *S. aureus* Nuc2 was clustered with the NUC sequences from 20 strains within the genus *Staphylococcus*, whereas *S. aureus* Nuc1 was out of the *Staphylococcus* cluster, clustered with other Gram-positive species such as *L. monocytogenes* and *Enterococcus faecalis*. Besides, in the phylogenetic clusters of the *S. aureus* Nuc2 group, the NUC sequence differentiated staphylococci similar to taxonomy classification based on 16S rRNA sequences (Takahashi et al. 1999). For example, eight various species within the *Staphylococcus hyicus-intermedius* group were clustered together (Fig. 2).

3.2. Recombinant expression of Nuc1 and Nuc2

Recombinant Nuc1 expression in *E. coli* showed three strong bands of nuclease activity in the zymogram gel (Fig. 3a). This result correlated well with the presence of three forms of staphylococcal nuclease (Nuc1), one primitive form and two secretive forms (Kovacevic et al. 1985). On the other hand, recombinant Nuc2 had only one weak band of nuclease activity at an approximate mass of 20 kDa, although the corresponding induced protein band of Nuc2 on the SDS-PAGE was at a much higher level than that of Nuc1 (Fig. 3b). Furthermore, Nuc2 exhibited an approximate five-fold weaker activity than that of Nuc1 in the time course nuclease reaction at 30 min after incubation (Fig. 3c). Various types of nucleic acids, including genomic DNA from different bacteria, the circular dsDNA pBR122, pNuc, and total RNA from *S. aureus* were tested to evaluate substrate specificity of Nuc1 and Nuc2, respectively. Cleavage of closed circular (plasmid or phage) DNA is one of the main characteristics of endonucleases (Rangarajan and Shankar 2001). Both enzymes could degrade all types of nucleic acids without preference, indicating that Nuc2 was also a sugar non-specific endonuclease like Nuc1. However, Nuc2 was relatively less efficient in digesting small fragments of DNA/RNA compared with Nuc1 (data not shown).

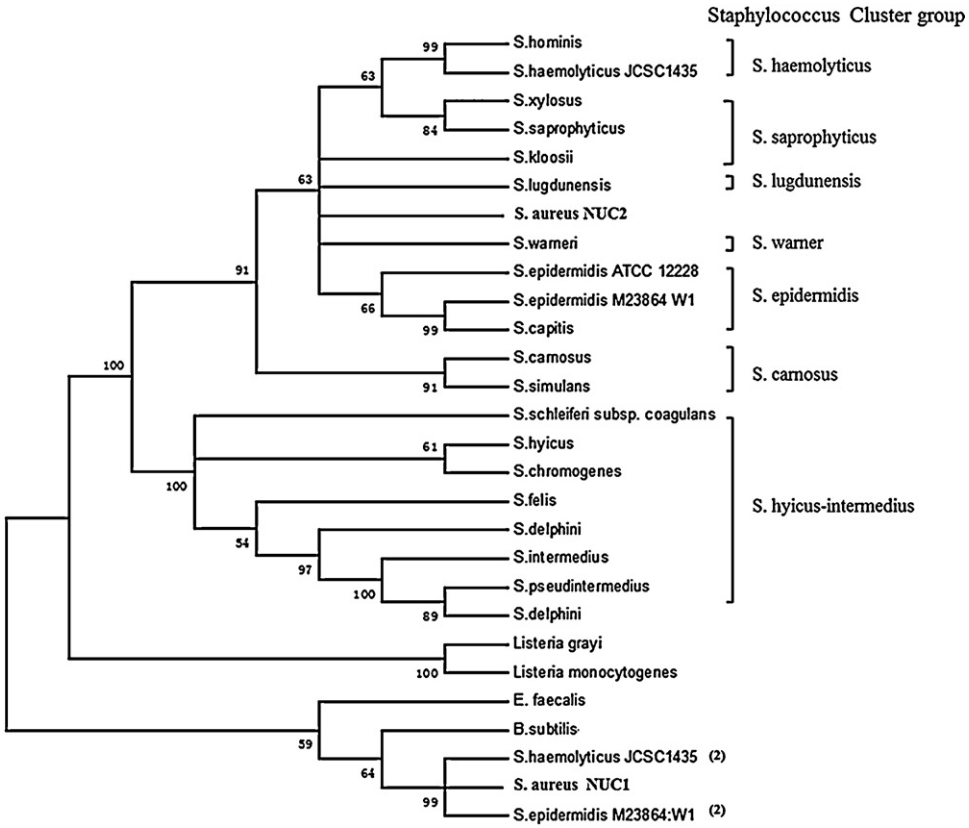

Fig. 2. Phylogenetic analysis of thermonucleases. Multiple alignments were based on 28 amino acid sequences from 24 different bacterial species, of which 20 species belonged to the genus *Staphylococcus*. Phylogenetic analysis was performed using the neighbor-joining method of MEGA 4.0. Bootstrap support values (1000 replicates) are shown next to branches. The consensus tree was drawn by a cut-off value 50%. The gi number of the sequences are the following: *S. epidermidis* M23864:W1 gi|242348880|, *S. epidermidis* M23864:W1(2) gi|242348589|, *S. warneri* L37603 gi|239597363|, *S. hominis* SK119 gi|228270938|, *S. capitis* SK14 gi|222443422|, *S. epidermidis* ATCC 12228 gi|27315467|, *S. xylosus* gi|226490488|, *S. carnosus* TM300 gi|222421057|, *S. kloosii*gi|226490490|, *S. haemolyticus* JCSC1435 gi|68447308|, *S. lugdunensis* HKU09-01 gi|289180420|, *S. saprophyticus* ATCC 15305 gi|72495268|, *S. simulans* gi|226490492|, *S. intermedius* gi|149689527|, *S. pseudintermedius* gi|149689525|, *S. hyicus* gi|226490494|, *S. delphini* gi|149689529|, *S. delphini* gi|209447007|, *S. felis* gi|226490500|, *S. schleiferi* subsp. coagulans gi|226490498|, *S. chromogenes* gi|226490496|, *E. faecalis* V583 gi|29345389|, *Listeria grayi* DSM 20601 gi|229310767|, *Listeria monocytogenes* str. 4b H7858 gi|47019024|, *B. subtilis* gi|1750126|, *S. haemolyticus* JCSC1435(2) gi|68447413|.

3.3. Effects of temperature and pH on the activity of both Nuc1 and Nuc2

Effects of temperature on the activity of the two nucleases were measured by an enzymatic activity assay at temperatures ranging from 20 to 80 °C (Fig. 4a). The optimum temperature for Nuc2 was 50 °C, and it was 55 °C for Nuc1. A lower percent relative activity of Nuc2 at 55, 60, and 70 °C was observed compared to Nuc1. These results indicated that there was less heat tolerance of Nuc2 compared to Nuc1. The pH effects were examined under the optimum temperature for each nuclease (Fig. 4b). Nuc2 exhibited optimal activity at pH 10, while the optimal pH for Nuc1 was at pH 9–10. This was in agreement with a previous report for staphylococcal nuclease (Tucker et al. 1978).

3.4. Effects of metal ions, enzyme inhibitors and other compounds on thermonuclease activity

The effects of different metal ions on the thermonuclease activity of Nuc1 and Nuc2 are shown in Fig. 5a. The thermonuclease activity of Nuc2 was stimulated in the presence of Ca^{2+} (0.05 mM) and Mg^{2+} (0.5 mM) up to 120% of its intrinsic activity and slightly increased in the presence of Co^{2+} (5 mM) and Ni^{2+} (0.05 mM). Nuc1 only exhibited a very small increase in thermonuclease activity in the presence of Ca^{2+} (0.05 mM), Mg^{2+} (0.05 mM), Co^{2+} (0.5 mM) and Ni^{2+} (0.05 mM). Metal ions Zn^{2+} and Mn^{2+} at 5 mM concentration showed sharp inhibition on both Nuc1 and Nuc2, retaining less than 40% of their initial activity. However, Nuc2 activity decreased with an increase in Zn^{2+} and Mn^{2+} concentrations while Nuc1 activity dropped sharply at 5 mM concentration. An increased activity of Nuc2 with Co^{2+} (5 mM) was observed while Nuc1 only retained 79% of its activity at this concentration.

The effects of metal chelators, thiol reagents, and detergents on Nuc1 and Nuc2 activity are shown in Fig. 5b. The thermonuclease activity of Nuc1 and Nuc2 increased when DTT, 2-ME, TritonX-100, and Tween-20 were added. In particular, Nuc1 activity was significantly enhanced by 1 mM DTT to 163.4%, and the activity of Nuc2 was increased with 1% (w/v) TritonX-100 to 210.5% of its initial activity. Moreover, Nuc2 activity was stimulated by 1 mM and 10 mM urea to approximately 117–124% while Nuc1 activity was not affected by urea. However, the thermonuclease activity of both Nuc1 and Nuc2 was reduced by the enzyme inhibitor EDTA and by the detergent SDS. There was 9% and 15% retained enzymatic activity for Nuc1 and 45% and 62% of Nuc2, respectively.

3.5. Comparative structural analysis of Nuc1 and Nuc2

To understand the difference of enzymatic activities between Nuc1 and Nuc2, active sites and protein domains were determined and compared using the InterPro database. The Nuc2 3D structure was also predicted and compared with the Nuc1 structure. It was shown that Nuc2 contained a similar domain composition as Nuc1, including an oligonucleotide/oligosaccharide-binding (OB)-fold, SNase (staphylococcal nuclease) domain, and three

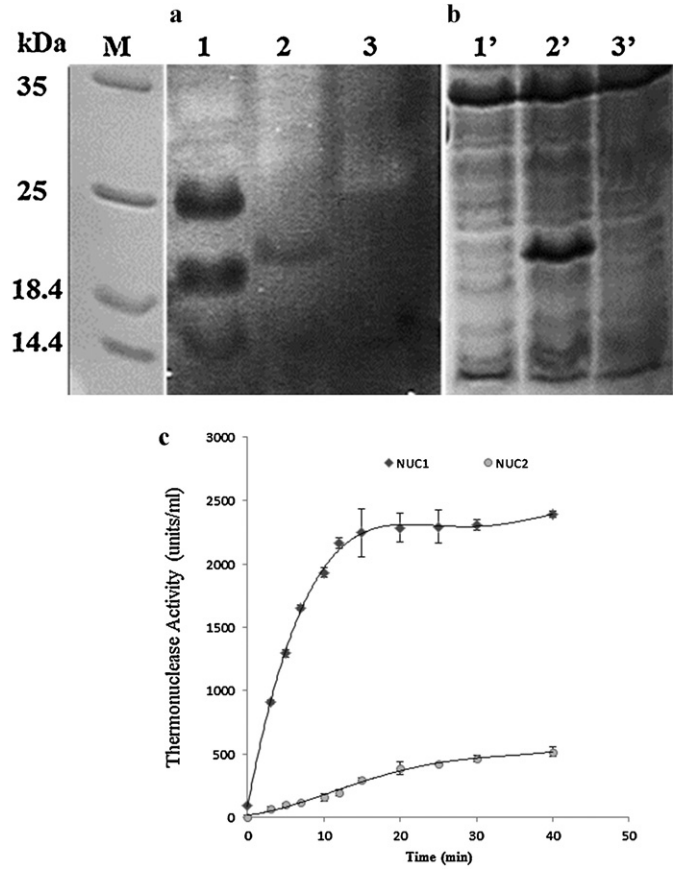

Fig. 3. Recombinant expressions of Nuc1 and Nuc2 in *E. coli*. (a) *In situ* nuclease activities shown using a zymogram gel. (b) SDS-PAGE analysis. Lane M: protein molecular weight marker; lane 1 and 1′: recombinant strain of *E. coli* BL21 containing the *nuc1* gene; lane 2 and 2′: recombinant strain of *E. coli* BL21 containing the *nuc2* gene; lane 3 and 3′: control strain of *E. coli* BL21 (pET28a). (c) Time course of nuclease reaction of recombinant Nuc1 and Nuc2.

conserved catalyzing active amino acid residues (Fig. 1). Superposition of Nuc1 and Nuc2 structures also showed no major differences (Fig. 6). However, Nuc2 did not fit with one of the key thermonuclease family signatures, pattern TNASE_1: PS01123 (PROSITE) D-G-D-T-[LIVM]-x-[LIVMC]-x(9,10)-R-[LIVM]-x(2)-[LIVM]-D-x-P-E, although only two amino acids (60F and 62A) in Nuc2 had changed. Moreover, several major hydrophobic core residues of staphylococcal nuclease (Nuc1), important for tight packing and thermostability of the protein structure were found changed in Nuc2. The six residues depicted as a ball-and-stick model in Fig. 6 (102V, 104L, 145V, 151I, 171I and 178V) in Nuc1 were close to each other in the tertiary structure. They could form an extensive van der Waals contact network among themselves and with neighboring residues (Chen et al. 2004; Chen and Stites 2004). A large database of the stability behavior of over 150 packing mutants at these six positions has been studied, and these residues from the major hydrophobic core of Nuc1 were demonstrated to be important in packing and stabilizing staphylococcal nuclease (Xie et al. 2007; Chen and Stites 2004). However, these key residues were changed to F, A, L, V, V, Y in Nuc2, respectively. Particularly, Leu 104 was changed to Ala, which has a smaller side chain compared with Leu, and Val 145, a hydrophobic residue in Nuc1, was replaced by a hydrophilic residue Tyr in Nuc2 (circled in Fig. 7). These two mutations could weaken the van der Waals interactions, as well as the hydrophobic interactions among the residues of the hydrophobic core in Nuc2, suggesting less folding stability and thermostability of the structure compared to Nuc1.

3.6. Effects of nuc gene mutation on thermonuclease activity in S. aureus

To further understand the individual role that the *nuc1* and *nuc2* genes may play in *S. aureus*, a *nuc1* and *nuc2* double deletion mutant was constructed and the complementation of Nuc1 and Nuc2 was carried out using Nuc1-expressing and Nuc2-expressing plasmids. All of the deletion mutants had no obvious difference in growth rate compared with the wild type when cultured in TSB medium overnight. The changes in thermostable nuclease activity in the single and double deletion mutants and the complementation strains were tested on toluidine blue-DNA agar plates (Fig. 7). Compared to the parental strain, RNΔ*nuc1* and RNΔ*nuc2* exhibited reduced activity zones on the plate. The thermonuclease activity of the RNΔ*nuc2* was higher than that of the RNΔ*nuc1* on the toluidine blue-DNA agar. No activity was observed in the *nuc1* and *nuc2* double deletion mutant, suggesting that only *nuc1* and *nuc2* genes contribute to the thermonuclease activity. The complementation

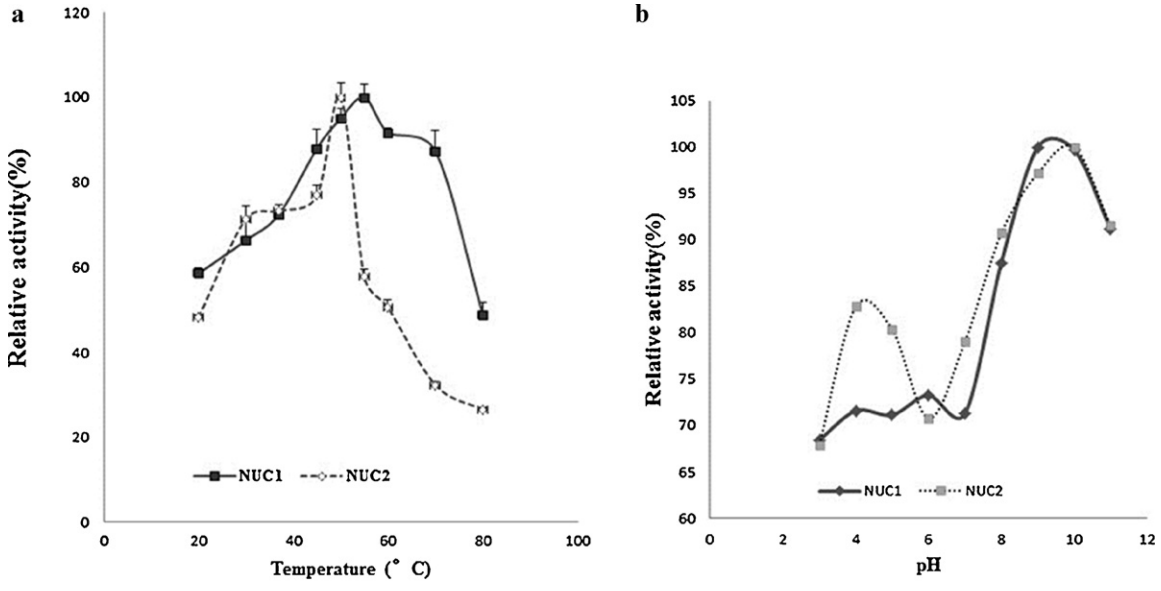

Fig. 4. Effects of temperature (a) and pH (b) on thermonuclease activity of Nuc1 and Nuc2.

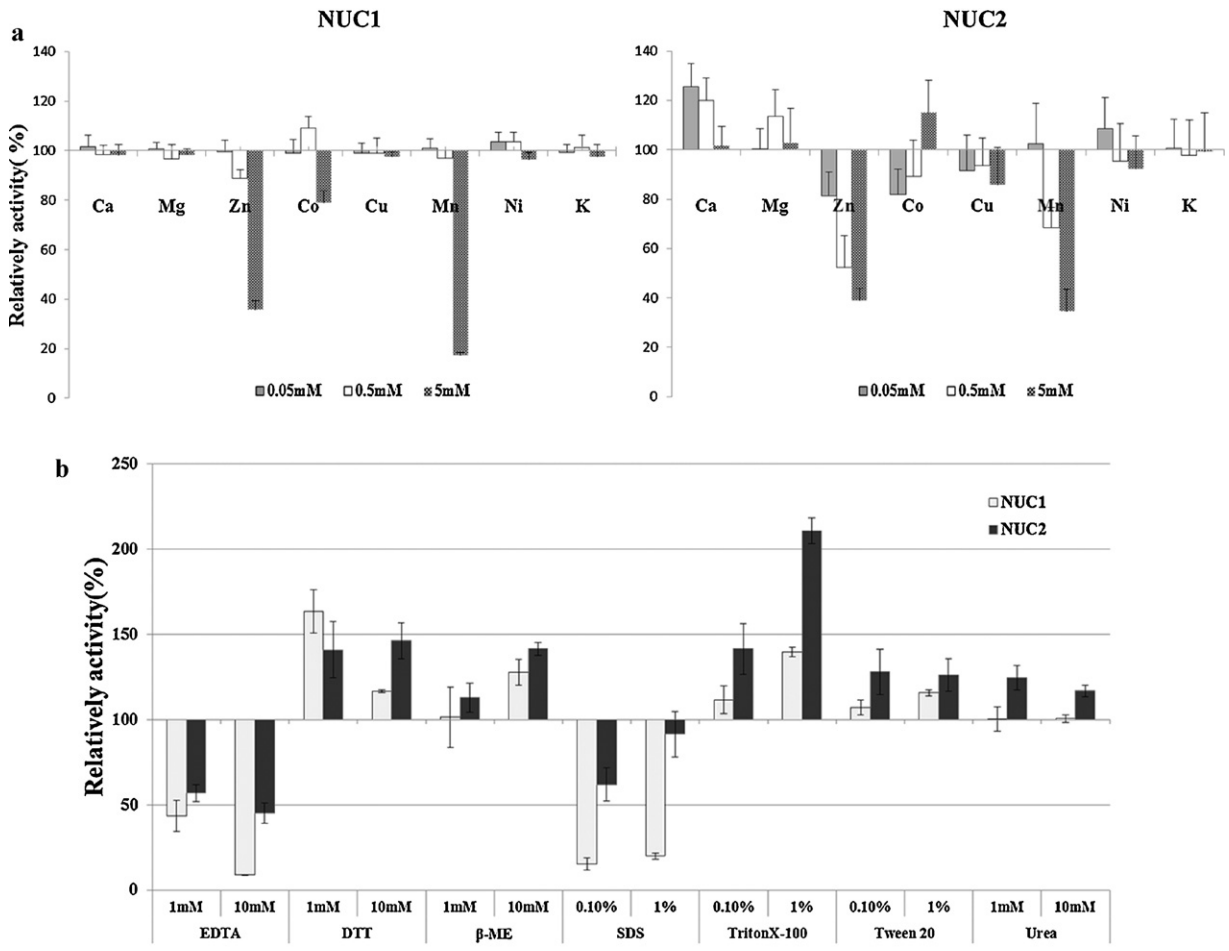

Fig. 5. Effects of metal ions (a), metal chelators, and other compounds (b) on thermonuclease activity. The concentrations are represented as the levels of the compounds in the incubation solutions. The error bars display the standard deviation based on three replicates.

of the *nuc1* gene restored the entire nuclease activity in the double deletion mutant, while the complementation of *nuc2* could only restore the original *nuc2* activity as that of RNΔ*nuc1* on the toluidine blue-DNA agar. Although both genes were able to complement the thermonuclease activity of the double deletion mutant, the *nuc1* gene showed stronger activity than that of *nuc2* in *S. aureus*.

4. Discussion

The *nuc* genes annotated by homology comparison to thermonuclease were almost ubiquitous in the genus *Staphylococcus*. Current reports showed that regardless of their thermonuclease activity, all of the staphylococci investigated except for the *Staphylococcus sciuri* group carried *nuc* genes (Sasaki et al. 2007), many of which were recently sequenced (Hirotaki et al. 2011). In our study, twenty amino acid sequences of thermonucleases from different *Staphylococcus* species shared more than 70% similarity with *S. aureus* Nuc2 but less than 60% similarity with *S. aureus* Nuc1. Pairwise comparisons indicated that the amino acid sequence of *S. aureus* Nuc2 had a high homology with that of *S. epidermidis*, *Staphylococcus lugdunensis*, and *Staphylococcus hominis* (89.3%, 85.6% and 84.0%, respectively). Additionally, phylogenetic analysis of Nuc2 cluster was analogous for the most part to classification based on the 16Sr RNA gene (Fig. 2). These results revealed that the *S. aureus* Nuc2 was relatively conserved in the genus *Staphylococcus* while Nuc1 nuclease might have been derived from another evolutionary

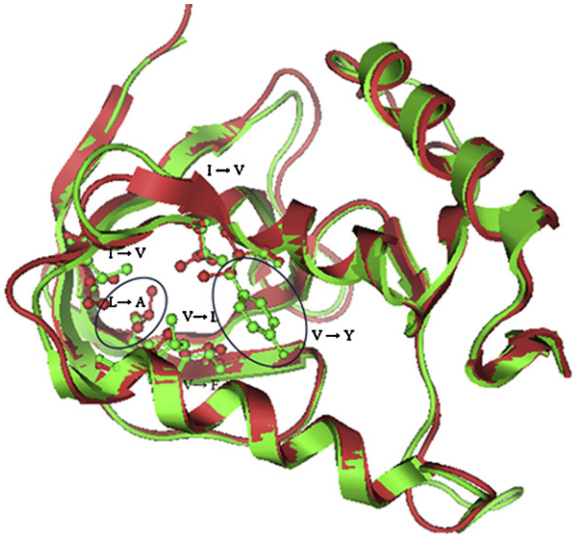

Fig. 6. Superposition of Nuc1 and Nuc2 structures. The Nuc1 crystal structure is shown in red cartoon form, and the predicted structure of Nuc2 is colored in green. The 6 major residues of the hydrophobic core of staphylococcal nuclease are shown as ball-and-stick models, and the corresponding amino acid changes of Nuc2 are shown near the marker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

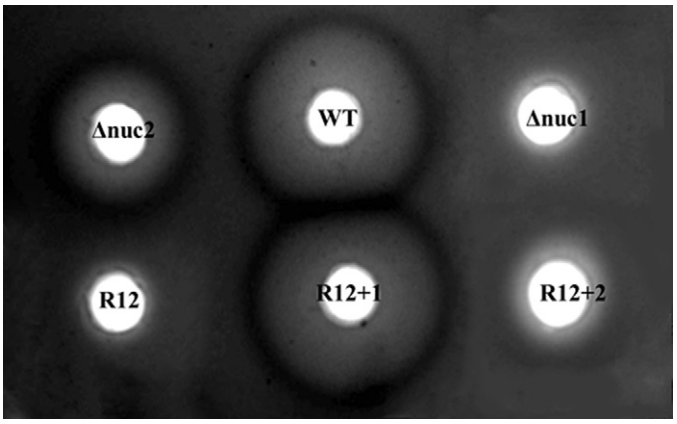

Fig. 7. Toluidine blue-DNA assays for thermonuclease activity of the *nuc* mutants and the complementary strains in *S. aureus*. The thermonuclease of the parental strain RN4220 (WT), *nuc1* deletion mutant (Δnuc1), *nuc2* deletion mutant (Δnuc2), *nuc1* and *nuc2* double deletion mutant (R12), *nuc1* complementary strain of double mutant (R12+1) and *nuc2* complementary strain of double mutant (R12+2) were assessed using Toluidine blue-DNA assays. The clear halo around the hole designates the activity of each sample.

branch out of the *Staphylococcus* group possibly through horizontal transfer. Such lateral transfer of pathogenicity islands between *S. aureus* and *L. monocytogenes* has been observed recently (Chen and Novick 2009). The exchanges among *Staphylococcus* group, particularly among *S. aureus*, *S. epidermidis*, and *Staphylococcus haemolyticus* have also been identified by whole genome sequencing (Watanabe et al. 2007; Becker et al. 2007). Furthermore, a new predicted thermonuclease was identified on a single mobile island phage φSPβ (*Bacillus subtillis* φSPβ like phage) in one *S. epidermidis* strain (RP62a), which is unique to this strain, and transfer was likely a recent event (Gill et al. 2005). However, two *nuc* genes were found in almost all strains of *S. aureus.* It is possible that *S. aureus nuc1* was acquired by lateral transfer from another pathogenic species in an earlier event and became stably integrated into the genome of *S. aureus*, making it a more-virulent member within the genus *Staphylococcus*.

The clear bands visualized after DNA hydrolysis on the zymogram gel (Fig. 3a) indicated a successful recombinant expression of *nuc1* and *nuc2* in *E. coli*; however, a relatively higher level of the recombinant Nuc2 protein revealed a fivefold weaker corresponding activity compared with that of Nuc1 (Fig. 3). These results indicated that the catalytic activity of Nuc2 nuclease was lower than that of Nuc1. The weaker nuclease activity of Nuc2 may due to the lack of a consensus pattern associated with the thermonuclease activity in the sequence of Nuc2 nuclease. Moreover, the comparative analysis of Nuc2 revealed detailed differences between Nuc1 and Nuc2 nuclease. Only 32% of Nuc2 activity remained at 70 °C while the Nuc1 enzyme maintained 87% of its activity at this temperature (Fig. 4a). These differences corroborated the prediction that changes in the hydrophobic core residues in Nuc2 reduced the interactions within the hydrophobic core and impact the folding stability and thermostability compared to Nuc1 (Fig. 6). Similarly, it was found that additional Ca^{2+} and Mg^{2+} stimulated the activity of Nuc2 up to 120% of its intrinsic activity while additional Ca^{2+} and Mg^{2+} barely affected the Nuc1 enzyme activity (Fig. 5a). Calcium ion is known as a cofactor to stabilize the enzyme structure in the absence of disulfide bonds (Tucker et al. 1978). These results suggested that the Nuc2 protein required more metal ions to stabilize the enzymatic structure compared with Nuc1. Additionally, compared with Nuc1, Nuc2 was more sensitive to the potent inhibitors with a higher molecular weight such as Zn^{2+} and Mn^{2+}(Fig. 5a) also implying less stability of the Nuc2 structure compared to Nuc1.

Furthermore, to determine the respective contribution of each *nuc* gene to the overall thermonuclease activity in *S. aureus*, phenotypic thermonuclease characterization was carried out after construction of *nuc* deletion mutants in *S. aureus* strain RN4220 and its complemented strains (Fig. 7). Thermonuclease activity was substantially decreased in RNΔ*nuc1* compared with the parental strain, while RNΔ*nuc2* retained major thermonuclease activity as assessed using toluidine blue-DNA agar. Likewise, the *nuc1* gene expressed from the complemented plasmid could fully restore the activity of the double deletion mutant to the wild type level while *nuc2* gene expression could only restore the original *nuc2* activity in RNΔ*nuc1nuc2* as that of the RNΔ*nuc1*. These results demonstrated that Nuc1 played a predominant role in total thermonuclease activity of *S. aureus* RN4220. This is most likely due to the lower catalytic activity of Nuc2 compared with Nuc1 (Fig. 3). Besides, the higher mRNA level of the *nuc1* gene at the post-exponential growth phase than that of *nuc2* in *S. aureus* could be another possibility for the variation in their functions (Hu et al. 2012).

Sequence alignment and phylogenetic analysis provided a discriminatory picture of the evolutionary relationship of *nuc2* and *nuc1*, in which Nuc2 was found to be more conserved within the genus of *Staphylococcus* and could potentially be used as a target for the identification of these species. The characterization of recombinant Nuc2 demonstrated that it was a different thermostable sugar non-specific nuclease in *S. aureus* with weaker thermonuclease activity and lower thermostability compared to Nuc1, which showed a correlation with the comparative structural prediction. Furthermore, the thermonuclease characterization of *S. aureus nuc* mutants and the complementation demonstrated the major contribution of *nuc1* gene to the overall thermonuclease activity of *S. aureus.*

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